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# Pepsin-induced hydrolysis and coagulation of proteins in goat, sheep and cow milk



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## ABSTRACT

The kinetics of pepsin-induced  $\kappa$ -casein hydrolysis and coagulation in cow, goat, and sheep milk were investigated at 37 °C, pH 6.3 and 6.0. At a 0.1 U mg<sup>-1</sup> pepsin-to- $\kappa$ -casein ratio, sheep milk showed the fastest  $\kappa$ -casein hydrolysis, followed by cow and goat milk as assessed by quantifying the release of para- $\kappa$ -casein using RP-HPLC. Sheep milk coagulated most rapidly, with  $\kappa$ -casein hydrolysis of 64% and 59% at pH 6.3 and 6.0. Goat milk required higher levels of  $\kappa$ -casein hydrolysis (90% and 86% at pH 6.3 and 6.0), before coagulation could occur. Sheep milk formed denser curds, whereas goat milk curd had a more porous structure. Additionally, small-angle neutron scattering showed differing rates of aggregate size growth among species. These findings indicate  $\kappa$ -casein hydrolysis and coagulation differences are not just due to casein content but also physicochemical characteristics such as casein micelle size. This study deepens our understanding of sheep and goat milk coagulation mechanisms compared to cow milk during the early stages of gastric digestion.

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## 1. Introduction

For nutritional value and health benefits, milk from non-cow species such as buffalo, goat and sheep has been widely accepted by consumers (Claeys et al., 2014; Roy, Ye, Moughan, & Singh, 2020a). There are distinct differences in physicochemical characteristics amongst cow, goat and sheep milk (Park, Juárez, Ramos, & Haenlein, 2007). It is known that the fat, protein, and total solids content of sheep milk is higher than that in cow and goat milk. Milk from different species all contain  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ -, and  $\kappa$ -casein, albeit with slight variations in their relative proportion and amino acid sequences (Selvaggi & Tufarelli, 2012). For instance, compared with cow and sheep milk caseins, goat milk caseins contain less  $\alpha_s$ -casein, particularly  $\alpha_{s1}$  (Li, Delger, Dave, Singh, & Ye, 2022a; Park, 2007). The casein micelle size of cow, sheep and goat milk also differs. Casein micelle diameters of 142–229 nm, 173–207, and

220–270 nm have been reported for cow, sheep and goat milk, respectively (Pellegrini, Remeuf, & Rivemale, 1994; Pierre, Michel, Le Graët, & Zahoute, 1998; Remeuf, 1993; Remeuf, Cossin, Dervin, Lenoir, & Tomassone, 1991). In addition, the size also depends upon the stage of the lactation cycle (Day, Raynes, Leis, Liu, & Williams, 2017; de Kruif & Huppertz, 2012). However, the internal structure of casein micelles amongst different species of milk shows strong similarities (Ingham, Smialowska, Kirby, Wang, & Carr, 2018; Yang et al., 2023b).

Coagulation of milk is a fundamental process that occurs in both the manufacture of dairy products (e.g., yoghurt, cheese) (Lucey, 2002) and in the digestion of milk products (Huppertz & Chia, 2021; Ye, 2021). In the presence of digestive enzymes (e.g., chymosin and pepsin),  $\kappa$ -casein on the surface of casein micelles is hydrolyzed, resulting in coagulation of casein micelles. Compared to cow milk, a shorter coagulation time and weaker curd consistency of goat milk during chymosin-induced coagulation have been observed, which has been explained by a lower casein content, lower  $\alpha_{s1}$ -casein content and larger casein micelle size (Park et al., 2007; Remeuf & Lenoir, 1986). With a higher  $\beta$ -/ $\alpha_s$ -casein ratio,

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sheep milk is very sensitive to chymosin and tends to coagulate faster than cow or goat milk (Muir, Horne, Law, & Sweetsur, 1993; Park, 2007). In addition, sheep milk generally produces firmer curds and has a slower rate of syneresis due to a higher casein and colloidal calcium content (Muir et al., 1993; Storry & Ford, 1982).

During *in vivo* and *in vitro* gastric digestion, regardless of dairy species (i.e., cow, goat and sheep), milk coagulates and separates into curd and liquid phases (Li et al., 2022c; Pan et al., 2021; Roy et al., 2022; Roy, Ye, Moughan, & Singh, 2021). However, the coagulation and digestion behavior are different amongst these species, e.g., the firmness of the sheep skim milk curd is higher than that of cow and goat milk curds at the end of gastric digestion (Li et al., 2022b). As a result, the stomach emptying time, nutrient bioavailability and the functional properties of the milk greatly depend upon the coagulation behavior of the milk during digestion (Huppertz & Chia, 2021; Ye, 2021). For example, the proteins in goat milk are hydrolyzed faster and more extensively than cow milk proteins (Rutella, Solieri, Martini, & Tagliacruzchi, 2016; Tagliacruzchi, Shamsia, Helal, & Conte, 2017).

It is known that the coagulation of milk at pH ~6.1 to 6.3 in the stomach is due to the action of pepsin (Huppertz & Chia, 2021; Tam & Whitaker, 1972; Ye, 2021). The kinetics of pepsin-induced hydrolysis of  $\kappa$ -casein and the consequent coagulation of cow milk have been extensively investigated (Yang et al., 2022). To measure the microstructure of milk curds, several techniques have been applied, including microscopy, diffusing wave spectroscopy, and (ultra) small-angle neutron scattering ((U)SANS). Neutron scattering methods are valuable tools for the study of food colloids (Gilbert, 2019). Indeed, according to previous research, (U)SANS has been shown as a powerful technique to probe the microstructure of milk curd induced either by acid, tamarillo protease, or rennet (Callaghan-Patrarachar, Peyronel, Pink, Marangoni, & Adams, 2021; Li et al., 2018; Yang et al., 2023b). However, information about the kinetics of pepsin-induced hydrolysis of  $\kappa$ -casein and the consequent coagulation of sheep and goat milk is limited.

Since casein is the main component in milk that forms the primary structure of the curd, the coagulation process of milk will greatly depend upon the kinetics of hydrolysis of  $\kappa$ -casein. To extend knowledge of pepsin-induced hydrolysis of  $\kappa$ -casein and the consequent coagulation of proteins in different milk (cow, sheep, and goat), raw fresh milk samples were treated with pepsin at pH 6.3 and 6.0. These two pH values were specifically selected because acid-induced coagulation occurs at pH < 5, and also hydrolysis of  $\kappa$ -casein and coagulation are affected by varying pH levels (Yang et al., 2022). As the  $\kappa$ -casein content differs in milk from different species, the kinetics of the pepsin-induced hydrolysis of  $\kappa$ -casein were investigated at the same pepsin-to- $\kappa$ -casein ratio (0.1 U mg<sup>-1</sup>) by measuring the release of para- $\kappa$ -casein using RP-HPLC. The consequent coagulation behavior and curd microstructures were examined by rheology and confocal laser scanning microscopy (CLSM), respectively. To exclude the effect of different coagulation behavior being related to the casein content in the different types of milk, cow, sheep, and goat milk samples were reconstituted to the same casein concentration (~2.3%). Subsequently, at the same pepsin activity, the structural changes of curds from different milk species were compared using SANS.

## 2. Materials and methods

### 2.1. Materials

Three different batches of raw whole milk were collected from milk suppliers between January and May 2022. The cow milk was collected from Massey University No. 4 dairy farm (Palmerston North, New Zealand), sheep milk was obtained from Fernglen Ltd.

(Masterton, New Zealand), and goat milk was collected from Central Dairy Goats Ltd. (Levin, New Zealand). Upon receipt of the milk samples, 0.02% (wt/wt) sodium azide (Merck KGaA, Darmstadt, Germany) was added to prevent microbial growth. The fat layer was removed by centrifugation of raw whole milk at 3000×g, 15 min, 4 °C with a Thermo Fisher Scientific Multifuge Heraeus 3SR+ centrifuge (Thermo Electron LED GmbH, Osterode, Germany). The initial pH of the skim milk samples was measured and then adjusted to 6.3 or 6.0 at 25 °C by the gradual addition of 1 M HCl under vigorous stirring conditions. The raw skim milk samples were stored at 4 °C for a maximum of 5 days before being utilized. Small pH adjustments were made to achieve the desired pH value, as required, before further experiments.

Porcine pepsin (EC 3.4.23.1; Sigma–Aldrich, St. Louis, MO, USA) with stated activity of 541 units per milligram of protein (U mg<sup>-1</sup>) was dissolved in Milli-Q water (5 mg per 5 mL; activity 541 U mL<sup>-1</sup>). All other chemicals (analytical grade) were obtained from Sigma–Aldrich unless otherwise specified.

### 2.2. Determination of milk composition

The composition of the cow, goat and sheep milk was analyzed using a Milkoscan (Foss, Hilleroed, Denmark). The ionic calcium concentration [Ca<sup>2+</sup>] in the milk samples was determined using a calcium-selective electrode (Orion 9720BNWP; Thermo Scientific, Waltham, MA, USA), as described by Yang et al. (2023a). The [Ca<sup>2+</sup>] of milk samples was measured at room temperature (~25 °C) before and after pH adjustment.

### 2.3. Particle sizes measurement by dynamic light scattering

The particle sizes of casein micelles in raw milk were measured by a Malvern Zetasizer (Nano ZS; Worcestershire, UK) using disposable cells (DTS0012) at 25 °C. The milk samples were diluted 50× with calcium imidazole buffer solution (20 mM imidazole, 5 mM CaCl<sub>2</sub>, 30 mM NaCl, pH 7.0) and analyzed under the 'milk protein size analysis' option (Anema & Li, 2003). The particle sizes of casein micelles were measured before and after pH adjustment. In addition, a Mastersizer Hydro 2000 (Malvern Instruments Ltd, Worcestershire, U.K.) was used to determine the specific surface area (m<sup>2</sup> g<sup>-1</sup>) of the casein micelles.

### 2.4. Pepsin-induced hydrolysis and coagulation at the same pepsin to $\kappa$ -casein ratio

To treat the three milk samples at the same pepsin to  $\kappa$ -casein ratio (0.1 U mg<sup>-1</sup>, hydrolysis and aggregation occurred within 90 min at this pepsin concentration), the pepsin solution (541 U mL<sup>-1</sup>) was diluted 7.7×, 6.3×, and 11.7× with water, respectively. The diluted pepsin solutions were added to milk at a ratio of 10  $\mu$ L per 1 mL milk. As a result, the final pepsin concentrations in cow, sheep and goat milk were ~0.7, 0.9 and 0.5 U g<sup>-1</sup>, respectively.

#### 2.4.1. Measurement of hydrolysis of $\kappa$ -casein

The action of pepsin on  $\kappa$ -casein was tracked by measuring changes in the amount of para- $\kappa$ -casein using RP-HPLC (Yang et al., 2022). An aliquot of diluted pepsin solution (20  $\mu$ L) was added to 2 mL samples, which was then immediately transferred into eight different test tubes (0.2 mL per tube) and incubated in a water bath at 37 °C. The reaction was stopped at different times (0, 2, 5, 10, 20, 30, 60, and 90 min) with HPLC buffer solution (0.8 mL, 6 M guanidinium-HCl, 0.1 M Bis-Tris, 19.5 mM DL-dithiothreitol and 5.37 mM sodium citrate, pH 7). Note, the sample at 0 min corresponds to the milk samples without pepsin addition. Each sample

was shaken for 10 s and left at room temperature for 1 h before HPLC injection. Four time points (5, 10, 30, and 90 min) were examined by HPLC in triplicate.

The analysis was carried out using a Nexera-X2 ultra-HPLC instrument equipped with an SPD-M20A diode array detector (Shimadzu, Kyoto, Japan). The protein composition of milk samples (0 min, without pepsin addition) was indicated by percentages based on the peak areas from the individual proteins to the total peak area of all proteins using LabSolutions Main (Shimadzu) software. The amount of para- $\kappa$ -casein was quantified based on the peak areas with an established method described previously (Yang et al., 2022). The degree of hydrolysis of  $\kappa$ -casein at each time point was thus determined, calculated as the ratio between the obtained chromatographic peak area of para- $\kappa$ -casein at a given time and the chromatographic peak area of para- $\kappa$ -casein at complete hydrolysis (Yang et al., 2022).

#### 2.4.2. Rheological measurements

The coagulation process was determined by measuring the storage modulus ( $G'$ ) as a function of time using a stress-controlled rheometer (MCR301 Anton Paar, Graz, Austria) equipped with a Couette geometry (Anton Paar; CC27, with 28.93 mm cup diameter and 26.64 mm bob diameter) (Yang et al., 2022). An aliquot of diluted pepsin solution (200  $\mu$ L) was added into 20 mL samples, i.e., the same ratio as used above – and stirred for 30 s before loading. By carrying out a time sweep measurement with a strain of 1% and a constant frequency 0.1 Hz at 37 °C for 90 min,  $G'$  was recorded every three minutes. The onset of coagulation (coagulation time) was defined as the intercept of a linear regression of the first four  $G'$  points that consistently increase above the time axis. The firming rate was defined as the maximum slope of  $G'$  as a function of time ( $dG'/dt$ ) (Yang et al., 2022).

After the time sweep measurement over 90 min, the large deformation properties of coagulum were determined by applying a constant shear rate of 0.00185  $s^{-1}$ . The yield stress was defined as the point when the shear stress started to decrease (Lucey, Teo, Munro, & Singh, 1997; Choi, Horne, & Lucey, 2007). All measurements were carried out in triplicate.

#### 2.4.3. Confocal laser scanning microscopy (CLSM)

The microstructure of the pepsin-induced coagulum was examined by CLSM (Yang et al., 2022). Fast Green fluorescent dye (1% wt/vol in water) was used to stain proteins (3  $\mu$ L/100  $\mu$ L). An aliquot of diluted pepsin solution was added into the pre-stained samples with the pepsin concentration as stated above. The sample-pepsin mixture was transferred to the cavity of a microscope glass slide, covered with a glass cover slip, and incubated at 37 °C for 90 min. The fluorescence images were observed using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with a 63 $\times$  magnification lens at room temperature.

#### 2.5. Small-angle neutron scattering (SANS)

Aliquots of raw skim milk from different species (mentioned in section 2.1, before pH adjustment) were freeze-dried. All freeze-dried milk powders were reconstituted into Milli-Q H<sub>2</sub>O to ~2.3 wt % casein content with gentle magnetic stirring for ~10 h to allow protein hydration at room temperature (~25 °C). The pH of the skim milk samples was then adjusted to 6.3 or 6.0 at 25 °C by the gradual addition of 1 M HCl under vigorous stirring conditions. The pepsin solution (541 U mL<sup>-1</sup>) was diluted with water and added to milk at a ratio of 10  $\mu$ L per 1 mL milk, resulting in a similar pepsin-to- $\kappa$ -casein ratio at 0.1 U mg<sup>-1</sup> (0.4 U g<sup>-1</sup> milk) for milk samples from all species. After shaking for 30 s, the samples at pH 6.0 were transferred into the

SANS sample cells and incubated in an oven at 37 °C for 2 h to reach equilibration. The samples at pH 6.3 were transferred into the SANS demountable sample cells and SANS measurements conducted on the QUOKKA beamline at the OPAL reactor (Lucas Heights, NSW, Australia) (Wood et al., 2018) for real-time measurements (averaged over 2 min intervals for 120 min). A thermally controlled sample changer was used to maintain the sample temperature at 37 °C. Measurements on all samples (milk sample without pepsin addition, equilibrated samples at pH 6.0, and samples at pH 6.3) covered a  $q$ -range from 0.0007 to 0.03  $\text{\AA}^{-1}$  where  $q$  is the magnitude of the scattering vector and is equal to  $(4\pi/\lambda) \sin(\theta)$ , and where  $\lambda$  is the wavelength and  $2\theta$  is the scattering angle. The configurations were (i) source-to-sample distance (SSD) = sample-to-detector distance (SDD) = 20 m, (ii) SSD = SDD = 8 m and (iii) SSD = 4 m and SDD = 1.3 m with 500 mm detector offset, using a  $\lambda$  of 5  $\text{\AA}$  with 10% resolution; an additional focusing optics configuration (iv) with the same SSD and SDD as (i) which was used to access lower  $Q_{min}$  with  $\lambda$  of 8.1  $\text{\AA}$  with 10% resolution. All data were reduced using IGOR Pro 8.02 software modified for the QUOKKA instrument with the scattering from H<sub>2</sub>O subtracted as background (Kline, 2006). Data fitting was carried out using SasView software (version 5.0.5, <https://www.sasview.org/>).

A Hamma–Porod model describing the radius of gyration (Hammouada, 2010), and the power-law behavior of a scattering system was used to fit the SANS data. The scattered intensity is given by:

$$I(q) = \frac{G}{q^S} \exp\left[\frac{-q^2 R_g^2}{3-S}\right], q \leq q_1$$

$$I(q) = \frac{D}{q^m}, q \geq q_1 \quad (1)$$

where  $q$  is the magnitude of the scattering vector,  $I(q)$  is the scattered intensity,  $R_g$  is the radius of gyration,  $m$  is the Porod exponent,  $G$  and  $D$  are the Guinier and Porod scale factors, respectively.  $S$  is equal to zero for spheres in this study. The Guinier form is used for  $q \leq q_1$  and the Porod form is used for  $q \geq q_1$ . Enforcing the continuity of the Guinier and Porod functions and their derivatives yields:

$$q_1 = \frac{1}{R_g} \sqrt{\frac{3m}{2}}$$

$$D = G \exp\left(\frac{-q_1^2 R_g^2}{3}\right) q_1^m = G \exp\left(-\frac{m}{2}\right) \left(\frac{-3m}{2}\right)^{\frac{m}{2}} \frac{1}{R_g^m} \quad (2)$$

### 3. Results and discussion

#### 3.1. Physicochemical properties of cow, sheep and goat milk

Differences in the physicochemical properties of cow, goat and sheep milk are listed in Table 1. The highest protein content, casein content, and total solids content were found in sheep milk, consistent with previous reports (Li et al., 2022a; Nguyen, Afsar, & Day, 2018; Roy, Ye, Moughan, & Singh, 2020b). The initial pH was similar for the three milk samples, at ~6.7. At the initial pH, the average casein micelle sizes were ~161, ~186, and ~210 nm for cow, sheep, and goat milk, respectively, in accordance with previous studies (Pellegrini et al., 1994; Pierre et al., 1998; Remeuf, 1993; Remeuf et al., 1991). The micelle size distributions can vary widely between different breeds and even between individual animals (de

**Table 1**  
Physicochemical properties of raw cow, goat and sheep skim milk.

	Cow milk	Sheep milk	Goat milk
Total solid, wt%	9.81 ± 0.27 <sup>Y</sup>	13.28 ± 0.62 <sup>X</sup>	8.08 ± 0.21 <sup>Z</sup>
Lactose, wt%	5.07 ± 0.26 <sup>X</sup>	5.09 ± 0.19 <sup>X</sup>	4.53 ± 0.14 <sup>Y</sup>
Fat, wt%	0.10 ± 0.10	0.43 ± 0.19	0.12 ± 0.14
Protein, wt%	3.90 ± 0.18 <sup>Y</sup>	6.31 ± 0.52 <sup>X</sup>	2.91 ± 0.12 <sup>Z</sup>
Casein, wt%	3.16 ± 0.04 <sup>Y</sup>	5.35 ± 0.06 <sup>X</sup>	2.29 ± 0.05 <sup>Z</sup>
κ-casein, wt%	0.56 ± 0.11 <sup>X</sup> (15%) <sup>a</sup>	0.69 ± 0.08 <sup>X</sup> (11%) <sup>a</sup>	0.37 ± 0.02 <sup>Y</sup> (13%) <sup>a</sup>
α <sub>s1</sub> -casein, wt%	1.15 ± 0.05 <sup>Y</sup> (33%) <sup>a</sup>	2.06 ± 0.17 <sup>X</sup> (36%) <sup>a</sup>	0.33 ± 0.01 <sup>Z</sup> (13%) <sup>a</sup>
α <sub>s2</sub> -casein, wt%	0.34 ± 0.02 <sup>Y</sup> (10%) <sup>a</sup>	0.77 ± 0.06 <sup>X</sup> (13%) <sup>a</sup>	0.33 ± 0.01 <sup>Y</sup> (13%) <sup>a</sup>
β-casein, wt%	1.39 ± 0.06 <sup>Y</sup> (40%) <sup>a</sup>	2.25 ± 0.19 <sup>X</sup> (39%) <sup>a</sup>	1.57 ± 0.07 <sup>Y</sup> (60%) <sup>a</sup>
Initial pH	6.70 ± 0.01	6.74 ± 0.06	6.70 ± 0.04
At initial pH			
Diameter, nm	161.0 ± 1.0 <sup>AZ</sup>	185.6 ± 4.0 <sup>AY</sup>	209.8 ± 10.0 <sup>AX</sup>
Specific surface area, m <sup>2</sup> g <sup>-1</sup> <sup>b</sup>	~48.8	~53.1	~35.0
[Ca <sup>2+</sup> ], mM	1.7 ± 0.3 <sup>CX</sup>	2.3 ± 0.6 <sup>CX</sup>	2.7 ± 0.1 <sup>CX</sup>
At pH 6.3			
Diameter, nm	160.8 ± 5.3 <sup>AZ</sup>	185.6 ± 2.5 <sup>AY</sup>	210.4 ± 13.3 <sup>AX</sup>
[Ca <sup>2+</sup> ], mM	5.5 ± 1.2 <sup>BX</sup>	6.2 ± 0.7 <sup>BX</sup>	5.3 ± 0.6 <sup>BX</sup>
At pH6.0			
Size, nm	159.2 ± 0.7 <sup>AY</sup>	190.8 ± 2.6 <sup>AX</sup>	199.5 ± 2.2 <sup>AX</sup>
[Ca <sup>2+</sup> ], mM	7.3 ± 1.0 <sup>AY</sup>	9.8 ± 0.9 <sup>AX</sup>	7.1 ± 0.1 <sup>AY</sup>
Pepsin concentration, U g <sup>-1</sup> milk	0.7	0.9	0.5
Pepsin-to-κ-casein ratio, U mg <sup>-1</sup>	0.1	0.1	0.1
Pepsin-to-casein surface area ratio, <sup>c</sup> U m <sup>-2</sup>	0.014	0.016	0.013

<sup>A–B</sup>Mean values for the same sample at different pH with different superscripts are significantly different ( $P < 0.05$ ). <sup>X–Z</sup>Mean values between samples at the same pH with different superscripts are significantly different ( $P < 0.05$ ). The results are expressed as the mean ± the standard deviation of the mean ( $n = 3$ ).

<sup>a</sup> The weight percent of each casein in total casein.

<sup>b</sup> Specific surface area (m<sup>2</sup>) per gram of casein micelles.

<sup>c</sup> Calculated as pepsin concentration divided by specific surface area.

Kruijff & Huppertz, 2012); in this study, the largest casein micelle diameter was found in goat milk, followed by sheep milk and cow milk, in agreement with Park et al. (2007). After pH adjustment, to pH 6.3 or 6.0, there was little change in casein micelle size.

There is no significant difference found in [Ca<sup>2+</sup>] between cow, sheep and goat milk at pH ~ 6.7 (Table 1). Due to the dissolution of colloidal calcium phosphate (CCP), higher [Ca<sup>2+</sup>] was observed at lower pH values for each species. The observed [Ca<sup>2+</sup>] in sheep milk was significantly greater than goat and cow milk at pH 6.0. Although the total calcium concentration was not measured in the current study, according to Li et al. (2022a) and Park et al. (2007), the total calcium concentration in sheep milk is greater than that in goat and cow milk; therefore, it can be concluded that more calcium transformed from the colloidal state to ionic state in sheep milk at pH 6.0.

The protein composition of milk samples (before the addition of pepsin) is shown in the HPLC pattern in Fig. 1A (black curves). A slight shift in retention times of the major casein peaks (α<sub>s1</sub>-, α<sub>s2</sub>-, β- and κ-) occurred in the cow, sheep and goat milk samples, which could be due to the protein genotypes (Li et al., 2022a). Based on the HPLC results, the casein compositions were calculated and presented in Table 1. Consistent with previous reports (Li et al., 2022a; Park et al., 2007), the most abundant protein in goat milk was β-casein and, compared to cow and sheep milk, α<sub>s1</sub>-casein was at the lowest level in goat milk. The calculated κ-casein in each milk was similar, at around 10–15% of casein proteins.

### 3.2. Pepsin-induced hydrolysis and coagulation

#### 3.2.1. The hydrolysis of κ-casein in cow, sheep and goat milk

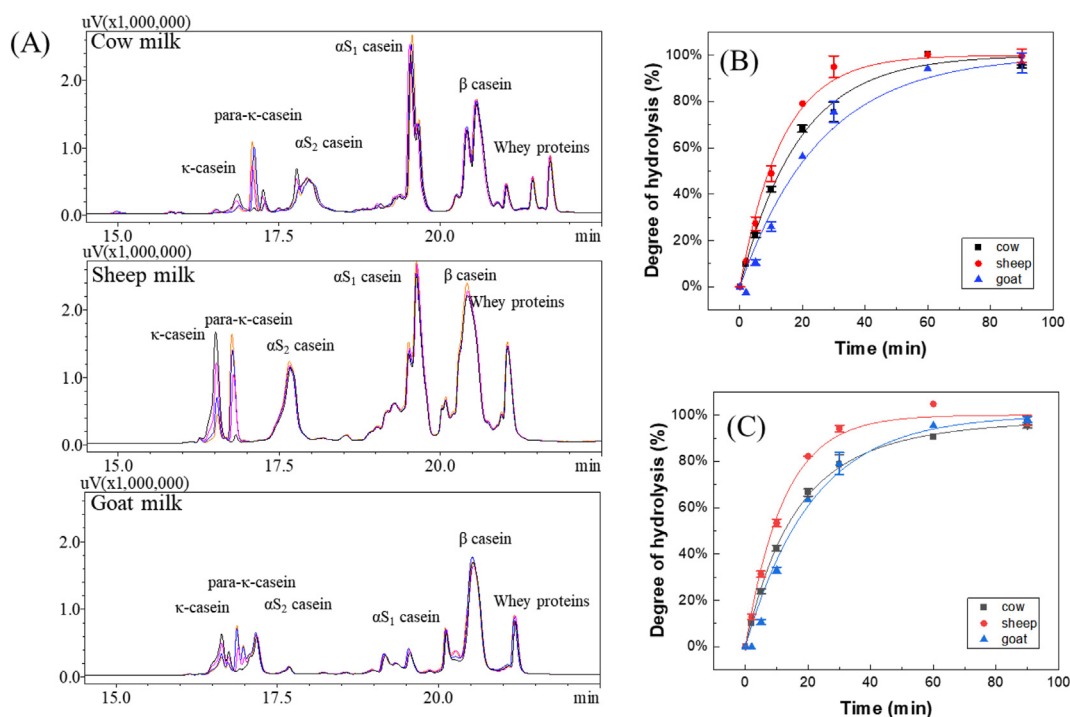
After the addition of pepsin, the peak area of κ-casein decreased and the para-κ-casein peak area increased, as shown in Fig. 1A. The hydrolysis degree of κ-casein increased with reaction time in the three milk samples at pH 6.3 and 6.0 as shown in Fig. 1B and C, respectively. The data were fitted to equation (3) that was

previously used for the pepsin-induced hydrolysis of κ-casein in cow milk (Yang et al., 2022):

$$\ln \left( 1 - \frac{H_t}{100} \right) = \frac{K_{enz} \cdot C}{K_{den}} \cdot [\exp(-K_{den} \cdot t) - 1] \quad (3)$$

where  $H_t$  is the percent hydrolysis of κ-casein at time  $t$ ,  $K_{den}$  (min<sup>-1</sup>) is the reaction rate constant for the denaturation reaction,  $C$  (U g<sup>-1</sup>) is the pepsin concentration,  $K_{enz}$  (min<sup>-1</sup> U<sup>-1</sup> g) is the reaction rate constant for the enzymatic reaction per unit quantity of enzyme solution, and  $K_{enz} \cdot C$  is defined as the overall reaction rate constant  $K$  (min<sup>-1</sup>).

The resulting reaction constants,  $K$  ( $K_{enz} \cdot C$ ), and denaturation constants,  $K_{den}$ , are shown in Table 2. The differences in  $K$  and  $K_{den}$  between pH 6.3 and 6.0 were not significant ( $P > 0.05$ ), regardless of the milk species. The  $K$  for sheep milk was significantly higher than cow and goat milk at either pH 6.3 or 6.0; this indicates that, at the same pepsin-to-κ-casein ratio, the hydrolysis reaction was fastest in sheep milk, followed by cow milk and goat milk. The reason for this difference is unclear, but it could be related to the genetic variations of κ-casein in different milk types. It has been reported that the Phe<sup>105</sup>-Met<sup>106</sup> bond, which is the specific bond hydrolyzed by pepsin, is present in all three milk types; however, according to Fox and Mulvihill (1990), it is the sequence around the Phe-Met bond, rather than the bond itself, that is the important determinant for hydrolysis. The alignment of the sequences of goat, sheep and cow κ-casein has been compared by Brignon, Chtourou, and Ribadeau-Dumas (1985) and differs in some of the amino residues, e.g., the assignment of residues from 101 to 105 are Thr-Thr-Leu-Ala-Arg, Thr-Ala-Met-Ala-Arg, and Thr-Thr-Met-Ala-Arg for goat, sheep and cow κ-casein, respectively. Although the total surface area to volume ratio is inversely related to the casein micelle size, the casein micelle content varies between samples. Assuming the casein micelles are spherical particles, the total surface areas (specific surface areas) of casein micelles were determined to be ~48, 53 and 35 m<sup>2</sup> g<sup>-1</sup> for cow, sheep and goat



**Fig. 1.** (A) Representative example of overlay of UV chromatograms analyzing protein profiles before (–) and after addition of pepsin into cow, sheep and goat milk at pH 6.3, (–) 30 min, (–) 60 min, (–) 90 min. (B) Degree of hydrolysis of  $\kappa$ -casein in each milk as a function of time after the addition of pepsin at pH 6.3. (C) Degree of hydrolysis of  $\kappa$ -casein in each milk as a function of time after the addition of pepsin at pH 6.0. The curves are fits to equation (3). Error bars represent SD from triplicate.

milk, respectively (Table 1). Assuming that the  $\kappa$ -casein is evenly located at the surface, for the same pepsin-to- $\kappa$ -casein ratio, the highest pepsin to casein micelle surface area ratio would be for sheep milk, followed with cow and goat milk (i.e.,  $\sim 0.014$ ,  $0.016$  and  $0.013 \text{ U m}^{-2}$  for cow, sheep and goat milk, respectively). Therefore, compared to cow milk, the higher pepsin to casein micelle surface area ratio in sheep milk should result in faster hydrolysis of  $\kappa$ -casein by pepsin; the lower pepsin to casein micelle surface area ratio in goat milk should result in a slower hydrolysis of  $\kappa$ -casein by pepsin. In addition, glycosylated  $\kappa$ -casein carries hydrophilic sugar moieties that both increase the negative charge and reduce the hydrophobicity of  $\kappa$ -casein (Holland & Boland, 2014; Vreeman, Both, Brinkhuis, & van Der Spek, 1977). According to Park et al. (2007), compared to 60% of  $\kappa$ -casein in cow milk, about 30% of  $\kappa$ -casein in sheep and goat are glycosylated. As pepsin (pI 2.6) is also negatively charged at both pH 6.3 and 6.0, the hydrolysis of  $\kappa$ -casein by pepsin could be more extensive in milk with higher glycosylated  $\kappa$ -casein due to reduced electrostatic repulsion between pepsin and glycosylated  $\kappa$ -casein. With these different properties of casein micelles in different milk species in mind, and based on the results in this study, the hydrolysis rate of  $\kappa$ -casein by pepsin followed the order: sheep milk > cow milk > goat milk.

### 3.2.2. The coagulation behavior of pepsin-induced cow, sheep and goat milk

After the addition of pepsin, the storage modulus ( $G'$ ) and loss modulus ( $G''$ ) as a function of time as measured by small oscillation rheology at pH 6.3 and 6.0 are shown in Fig. 2A and B, respectively. Consistent with Yang et al. (2022), for all milk species,  $G'$  showed a lag phase in the early stages; after the onset of coagulation,  $G'$  increased significantly and reached a plateau, or dropped slightly for some of the samples. This could be attributed to the syneresis of milk curd at a lower pH or a reduction in adhesion of the curd to the stainless steel in the rheometer. Values for the coagulation time, the firming rate ( $dG'/dt$ ), and  $G'_{max}$  are summarized in Table 3; all values were significantly affected by milk species ( $P < 0.05$ ).

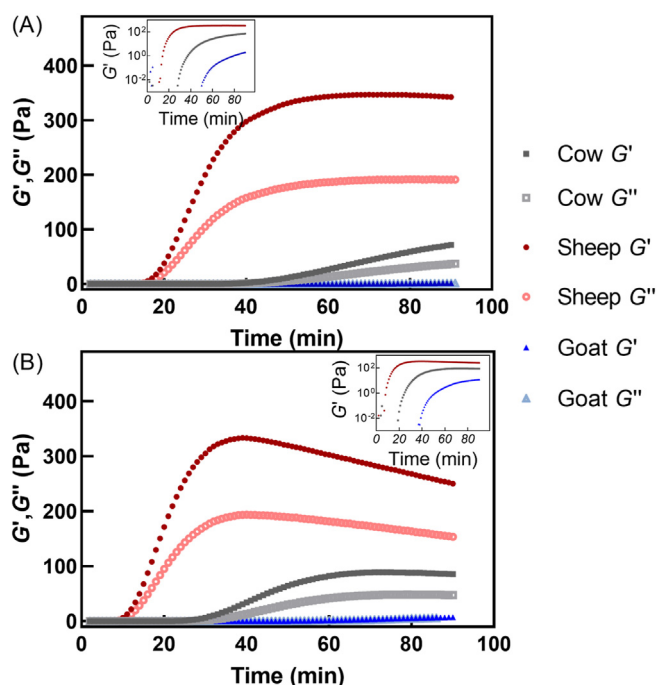
At both pH 6.3 and 6.0, shorter coagulation time was found in sheep milk, followed by cow milk and goat milk. By substituting the coagulation time into the hydrolysis equation, the hydrolysis degree at coagulation time was calculated and reported in Table 3. The cow milk coagulated when 81% of  $\kappa$ -casein was hydrolyzed at pH 6.3, and 67% at pH 6.0. The sheep milk coagulated when 64% of  $\kappa$ -casein was hydrolyzed at pH 6.3, and 59% at pH 6.0. For goat milk, the highest hydrolysis degree was required before coagulation could occur, corresponding to 90% and 86% hydrolysis at pH 6.3 and pH 6.0, respectively. Therefore, the shortest coagulation time for

**Table 2**

The hydrolysis kinetics of  $\kappa$ -casein in different systems after the addition of pepsin. Results are expressed as the mean  $\pm$  SD of the mean ( $n = 3$ ).

Sample	Hydrolysis kinetics of samples at pH 6.3 according to Eq. (3)		Hydrolysis kinetics of samples at pH 6.0 according to Eq. (3)	
	( $K_{enz} \cdot C$ ) ( $\text{min}^{-1}$ )	$K_{den}$ ( $\text{min}^{-1}$ )	( $K_{enz} \cdot C$ ) ( $\text{min}^{-1}$ )	$K_{den}$ ( $\text{min}^{-1}$ )
Cow milk	$0.059 \pm 0.003^{Ba}$	$0.01 \pm 0.01^{Aa}$	$0.056 \pm 0.001^{Ba}$	$1.19E-2 \pm 2.36E-3^{Aa}$
Sheep milk	$0.074 \pm 0.005^{Aa}$	$4.97E-9 \pm 5.59E-9^{Ba}$	$0.080 \pm 0.002^{Aa}$	$1.84E-9 \pm 1.31E-9^{Ba}$
Goat milk	$0.039 \pm 0.002^{Ca}$	$2.85E-9 \pm 3.97E-10^{Ba}$	$0.045 \pm 0.004^{Ca}$	$4.45E-9 \pm 5.55E-9^{Ba}$

<sup>a-b</sup>Mean values for the same sample at different pH with different superscripts are significantly different ( $P < 0.05$ ). <sup>A-C</sup>Mean values between samples at the same pH with different superscripts are significantly different ( $P < 0.05$ ). The results are expressed as the mean  $\pm$  the standard deviation of the mean ( $n = 3$ ).



**Fig. 2.** Evolution of  $G'$  (solid symbols) and  $G''$  (open symbols) for different milk types over time after the addition of pepsin at 37 °C: (A) pH 6.3 (B) pH 6.0, with an inset to show the  $G'$  plotted on a logarithmic scale.

sheep milk is not only due to the fast hydrolysis of  $\kappa$ -casein, but also the extent to which the sheep casein micelles are prone to aggregation, i.e., lower requirement in the amount of  $\kappa$ -casein being hydrolyzed for coagulation ( $H_{ct}$ ). The evolution of  $G'$  was also significantly different for cow, goat and sheep milk. The values of  $dG'/dt$  and  $G'_{max}$  of the sheep milk were higher than cow milk and goat milk at both pH 6.3 and 6.0.

The different coagulation behavior in different milk types could be related to the total solid content and casein content, which is associated with the number of crosslinks between casein micelles in the network. However, it was reported that even when casein concentrations or protein concentrations are equal, chymosin-induced goat curd is softer than its bovine counterparts (Miocinovic et al., 2016; Remeuf & Lenoir, 1986; Storry, Grandison, Millard, Owen, & Ford, 1983). This suggests that the specific properties of casein micelles from different species, such as the proportion of caseins fractions ( $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ -,  $\kappa$ -) (Fig. 1A), size of casein micelles (Table 1), and hydration could have a more significant effect on the coagulation of casein micelles than merely the quantity/content of the proteins (Park, 2007). According to

Dalgleish, Brinkhuis, and Payens (1981), small micelles start to aggregate at a slightly lower degree of proteolysis than large micelles. Therefore, one of the reasons for the highest requirement of  $H_{ct}$  for goat milk could be related to its largest casein micellar size. The lower proportion of  $\alpha_{s1}$ -casein in goat milk (13%) compared to that in cow (33%) and sheep (36%) milk has been reported to contribute to the weak gel properties in goat yoghurt (Miocinovic et al., 2016; Morgan et al., 2003; Nguyen et al., 2018), which could also be the case for pepsin-induced milk curd. As for sheep milk, even though the casein micelle size is larger than that of cow milk, the lowest requirement in  $H_{ct}$  could be related to the relatively higher total calcium concentration among the three milk types (Li, Delger, Dave, Singh, & Ye, 2023). It was reported that calcium promotes coagulation, inducing cross-linking and aggregation of para-casein micelles (Guinee, O'Brien, Law, & Tamime, 2010; Park, 2007). Besides, the average mineral content (e.g., Ca, Mg, etc.) in sheep milk is higher than that in cow and goat milk (Park, 2007). There is an inverse relationship between the mineralization of the micelle and its hydration, which also means that sheep milk is less hydrated than cow and goat milk (Remeuf & Lenoir, 1986; Sood, Gaiind, & Dewana, 1979). The rate of aggregation increased significantly under conditions where limited hydration of the casein micelles is expected to occur.

Comparing samples at pH 6.3 and 6.0, the coagulation time and required hydrolysis degree for coagulation were lower at a lower pH, which is in agreement with Yang et al. (2022). This is due to partial charge neutralization of the negatively charged para-casein micelles and the increased ionic calcium concentration at lower pH (van Hooydonk, 1987; Yang et al., 2022). After 90 min, large deformation testing was carried out on the milk curd; the associated yield stress data is summarized in Table 3. The highest yield stress was found for sheep milk, followed by cow milk and goat milk. The lowest yield stress for goat milk may be attributed to the occurrence of large pores and weaker interactions between casein particles (Lucey et al., 1997). The differences in firmness and the yield stress of the samples could be related to the different microstructures (Tamime, Kalab, Davies, & Younis, 1990), which will be discussed below.

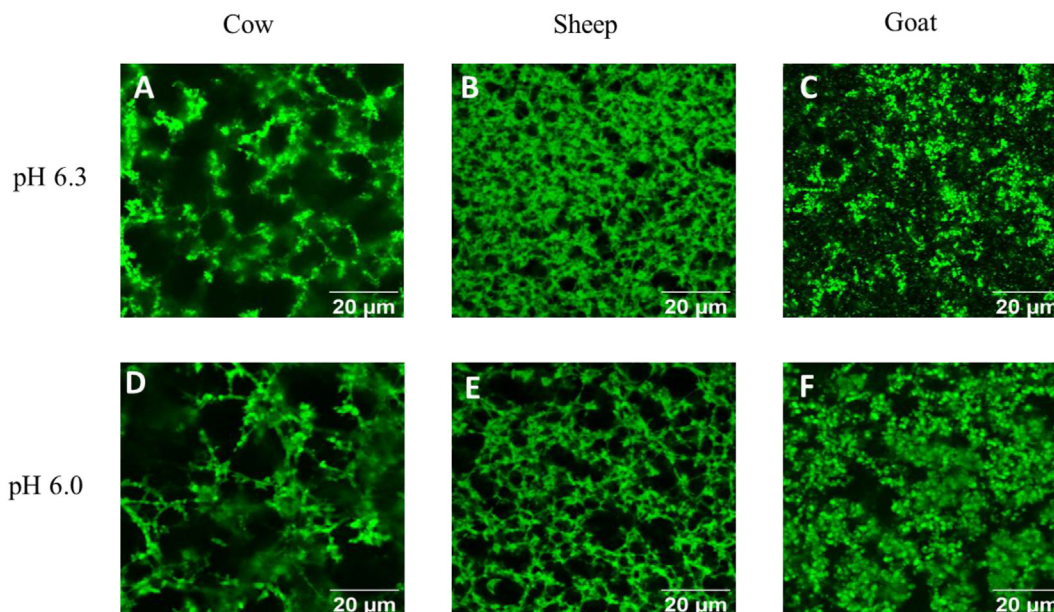
### 3.2.3. The microstructure of cow, sheep, goat milk curd

The microstructures of cow, goat and sheep milk curd were examined 90 min after the addition of pepsin (Fig. 3). The CLSM micrographs of all three curds consists of a protein network (appearing as green) and the pores (appearing as black). The protein network was different among cow, goat, and sheep casein curds. At both pH 6.3 and 6.0, the goat milk curd (Fig. 3C, F) exhibited the most porous microstructure and least dense protein network. Casein micelles in sheep and cow milk curd assembled into chains with obvious pores (Fig. 3A, B, D, E), with sheep milk

**Table 3**  
Coagulation properties of skim milk at pH 6.3 and pH 6.0 with the addition of pepsin.

		Cow milk	Sheep milk	Goat milk
pH 6.3	Coagulation time (min)	34.9 ± 7.2 <sup>Ba</sup>	13.6 ± 0.2 <sup>Ba</sup>	61.6 ± 13.1 <sup>Aa</sup>
	$H_{ct}$ (%)	81 ± 4 <sup>Ba</sup>	64 ± 3 <sup>Ca</sup>	90 ± 3 <sup>Aa</sup>
	Firming rate ( $dG'/dt$ , Pa min <sup>-1</sup> )	2.3 ± 0.6 <sup>Ba</sup>	18.0 ± 1.0 <sup>Aa</sup>	0.2 ± 0.2 <sup>Ca</sup>
	Maximum storage modulus ( $G'$ , Pa)	71.73 ± 32.6 <sup>Aa</sup>	350.00 ± 50.6 <sup>Aa</sup>	2.1 ± 3.1 <sup>Aa</sup>
	Shear stress (Pa)	16.5 ± 3.0 <sup>Ba</sup>	117.3 ± 129.7 <sup>Aa</sup>	1.0 ± 0.9 <sup>Ca</sup>
pH 6.0	Coagulation time (min)	22.2 ± 3.0 <sup>Ba</sup>	11.2 ± 1.2 <sup>Ca</sup>	44.2 ± 6.8 <sup>Ab</sup>
	$H_{ct}$ (%)	67 ± 4 <sup>Bb</sup>	59 ± 3 <sup>Ba</sup>	86 ± 3 <sup>Aa</sup>
	Firming rate ( $dG'/dt$ , Pa min <sup>-1</sup> )	3.7 ± 0.6 <sup>Ba</sup>	22.3 ± 1.2 <sup>Aa</sup>	0.3 ± 0.2 <sup>Ca</sup>
	Maximum storage modulus ( $G'$ , Pa)	89.8 ± 9.1 <sup>Aa</sup>	334.7 ± 14.5 <sup>Aa</sup>	7.3 ± 4.4 <sup>Aa</sup>
	Shear stress (Pa)	9.9 ± 2.0 <sup>Ba</sup>	47.4 ± 37.3 <sup>Ba</sup>	1.1 ± 0.3 <sup>Ba</sup>

<sup>a-b</sup>Mean values for the same sample at different pH with different superscripts are significantly different ( $P < 0.05$ ). <sup>A-C</sup>Mean values between samples at the same pH with different superscripts are significantly different ( $P < 0.05$ ). The results are expressed as the mean ± the standard deviation of the mean ( $n = 3$ ).



**Fig. 3.** Microstructures of the pepsin-induced cow, sheep and goat coagulum at 90 min at same pepsin-to- $\kappa$ -casein ratio as analysed by confocal laser scanning microscopy. The scale bars are 20  $\mu\text{m}$  in length for all micrographs. The proportion of black background area are ~63%, 27%, 63%, 63%, 53%, and 44% for A, B, C, D, E and F, respectively.

curd containing the most homogenous and compact structure with small pores (Fig. 3B, E). Compared to sheep milk curd, thicker protein strands were observed in cow milk curd (Fig. 3A, D). Limited studies have been carried out comparing the enzyme-induced microstructure of coagula in different skim milk types. According to Hamad, Ismail, and El-Menawy (2016) and Ong, Dagastine, Kentish, and Gras (2013), the dense protein structure of sheep cheese curd (chymosin-induced curd) could be due to the higher protein content. The high concentration of protein in the sheep milk samples decreases the mean free distance between casein micelles, resulting in a faster rate of coagulation and the development of a densely aggregated casein micellar network. The porous microstructure in goat curd illustrates the weak network which results in a lower  $G'$  value (Fig. 2).

Comparing the micrographs at pH 6.3 and 6.0, the average pore size observed for the cow and sheep milk curds at pH 6.0 was larger than that in the goat milk at pH 6.3. It has been reported that the rearrangement of the casein curd network occurs more extensively with decreasing pH (Mellema, Walstra, van Opheusden, & van Vliet, 2002) and facilitates syneresis. At later stages, a pronounced decrease in  $G'$  was consequently observed for sheep milk at pH 6.0 (Fig. 2B). For the goat milk, the curd formed a denser and more compact microstructure at pH 6.0 than at pH 6.3. This observation is consistent with rennet curd at different pH values by Ong et al. (2013). As shown in Fig. 2C,  $G'$  of the goat milk at pH 6.0 was higher than at pH 6.3, and kept increasing during 90 min of reaction, i.e., there was no drop in  $G'$  caused by syneresis.

### 3.3. SANS study of microstructures of different milk species with and without pepsin addition

To understand the changes in the internal structure of the casein micelles after the addition of pepsin, SANS was employed to probe the structure of samples reconstituted at the same casein content (Table 4). The dimensions of casein micelles in these samples were slightly different from those in native milk, but still follow the order: cow milk < sheep milk < goat milk. Although deuterated solvents are typically employed in neutron scattering measurements to facilitate contrast variation (Bayrak et al., 2021), according to

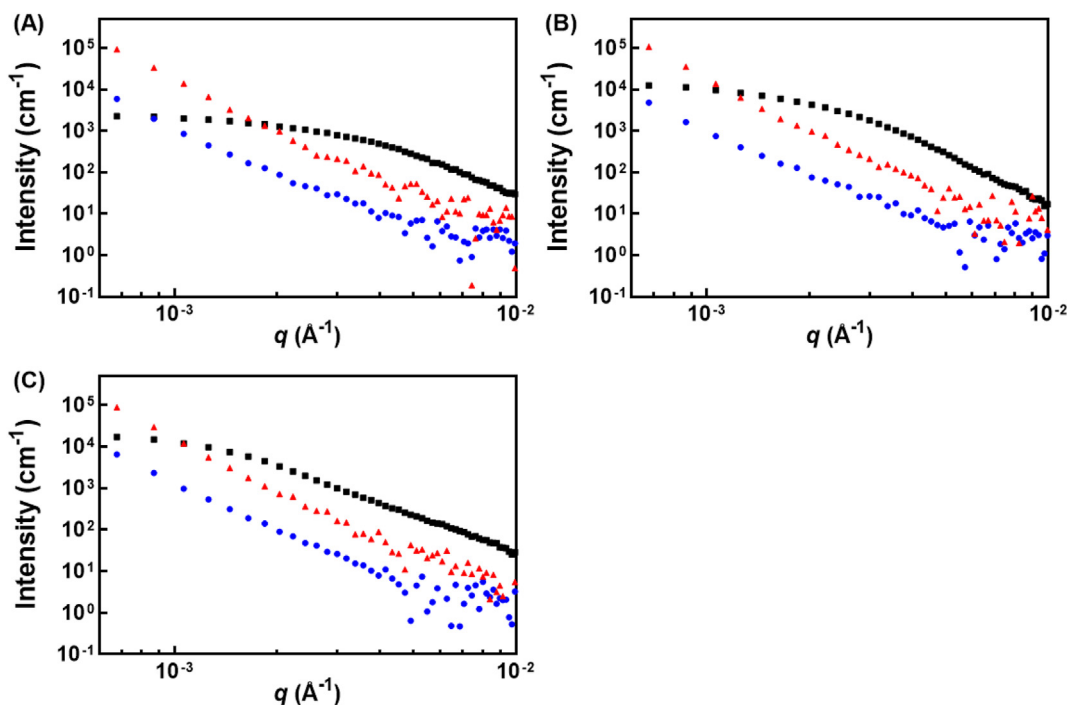
Lopez-Rubio and Gilbert (2009), the microstructure of casein curd in  $\text{D}_2\text{O}$  is markedly different than in  $\text{H}_2\text{O}$  dissolved samples. As a result,  $\text{H}_2\text{O}$  was used as a solvent in this study to avoid potential artefacts in the microstructure of milk proteins in these coagulated systems, as well as excluding the potential for solvent effects on the hydrolysis kinetics. Measurements covered a  $q$  range from ~0.0007 to 0.03  $\text{\AA}^{-1}$ , corresponding to a length scale of approximately 40–1000 nm and is thus complementary to the information obtained with CLSM.

Before the addition of pepsin, the SANS profiles of milk samples were measured, as shown in Fig. 4 (black curves). The curvature in the scattering as one approaches the lowest  $q$  is consistent with the presence of a Guinier region; this can be observed at  $q$  ~0.004  $\text{\AA}^{-1}$ , 0.003  $\text{\AA}^{-1}$ , and 0.0015  $\text{\AA}^{-1}$  for cow, sheep, and goat milk, respectively, and enables an assessment of the size of casein micelles (Gilbert, 2019; Holt, de Kruijff, Tuinier, & Timmins, 2003; Singh, Hemar, Gilbert, Wu, & Yang, 2020). The Guinier–Porod model was used to fit the curves (Fig. S1) and the fitting parameters are summarized in Table 5 (milk control). In this model, the casein micelles are regarded as spheres, and the hydrodynamic diameter  $D$  can be calculated using  $(D/2)^2 = 5/3 \times R_g^2$  (Witten, Pincus, & Weitz, 2005). Fits to each set of milk data gave a micelle diameter of ~129, ~201 and ~307 nm for cow, sheep and goat milk, respectively; these values are within the expected range from the literature (Day et al., 2017; de Kruijff, Huppertz, Urban, & Petukhov, 2012; Ingham et al., 2018; Yang et al., 2023b).

To investigate the changes in the structure of casein micelles as a function of reaction time at pH 6.3, SANS patterns obtained at 5, 10, 15, 20, 30, 60, 90 and 120 min after the addition of pepsin are provided in Fig. 5. As the reaction progressed, a significant increase in the scattering intensity was observed at  $q < 0.001 \text{\AA}^{-1}$ , indicating the formation and growth of large-scale aggregates. Guinier shoulder features can still be observed but are shifted to smaller  $q$  values, representing the formation of protein aggregates with dimensions of the order of  $q^{-1}$  (Li et al., 2018; Wang et al., 2019). Similar observations were found in previous studies of rennet (Callaghan-Patrarachar et al., 2021) and plant protease (tamarillin from Tamarillo fruits) (Li et al., 2018)-induced coagulation of cow milk. The curves obtained from 5 to 90 min were fitted with a

**Table 4**  
Physicochemical characteristics of different reconstituted milk used for small angle neutron scattering (SANS) studies. TS = total solids.

	Casein micelle size (nm)	TS (%)	Fat (%)	Lactose (%)	Protein (%)	Casein (%)	κ-casein (%)	Pepsin concentration (U g <sup>-1</sup> milk)	Pepsin-to-κ-casein ratio (U mg <sup>-1</sup> )
Cow milk	138	7.54	0.15	3.78	2.97	2.37	0.33	-0.4	-0.1
Sheep milk	158	6.24	0.32	2.46	3.06	2.39	0.27	-0.4	-0.1
Goat milk	209	8.35	0.37	4.51	3.00	2.30	0.30	-0.4	-0.1



**Fig. 4.** SANS data from initial milk samples (■) and equilibrated pepsin-induced coagulated samples (▲, pH 6.3; ●, pH 6.0), (A) cow; (B) sheep; (C) goat.

Guinier–Porod model, and the refined parameters are recorded in Table 5. The change of  $R_g$  (radius of gyration) was plotted as a function of reaction time in Fig. 6. Overall, the size of aggregates ( $R_g$ ) increases with reaction time; however, the kinetics differ between the species. A large increase in  $R_g$  was found in cow milk in the first 5 min, and a gradual increase was found from 5 min to 90 min; with a smallest aggregate size of the three species at 90 min; the diameter of aggregates in cow, sheep and goat milk curd were calculated to be 691, 1011 and 724 nm, respectively. The

aggregate size of goat milk showed little change in the first 20 min, then increased markedly from 30 min. The sheep milk kept increasing from 5 min to 90 min and grew to the largest size at 90 min. At 120 min, the Guinier feature, i.e., plateau region, was not observed, and the scattering intensity continued the increase in the low  $q$  region. A power law model was used to fit the curve at 120 min: the power law exponent at  $0.0006 < q < 0.015 \text{ \AA}^{-1}$  was  $\sim 3.80$ ,  $3.88$  and  $3.87$  in cow, sheep, and goat milk curd, respectively (Table 5). This is consistent with the casein micelles arranging as

**Table 5**  
Parameters obtained from the fit of the SANS experimental data (associated with Fig. 5) to Guinier–Porod equation and power law equation.

	Time (min)	Model	Cow			Sheep			Goat		
			Scale	$R_g$ (Å)	Porod/Power exponent	Scale	$R_g$ (Å)	Porod/Power exponent	Scale	$R_g$ (Å)	Porod/Power exponent
Milk control	0	Guinier Porod	1826(17)	501(3)	2.99(0.02)	10848(85)	779(3)	3.75(0.01)	19644(282)	1208(8)	2.93(0.01)
Milk at pH 6.3	5	Guinier Porod	28544(2121)	1580(50)	2.83(0.03)	14298(390)	954(12)	3.38(0.04)	27207(1096)	1416(26)	3.09(0.03)
	10	Guinier Porod	37887(2567)	1647(44)	3.00(0.03)	29818(1178)	1298(22)	3.16(0.03)	25526(946)	1332(22)	3.08(0.03)
	15	Guinier Porod	46407(3287)	1714(46)	3.15(0.03)	42311(2215)	1460(29)	3.46(0.03)	27738(986)	1317(21)	3.13(0.03)
	20	Guinier Porod	57874(4576)	1840(53)	3.21(0.03)	73395(4961)	1749(39)	3.69(0.04)	33474(1034)	1341(18)	3.32(0.03)
	30	Guinier Porod	71788(5961)	1895(54)	3.48(0.03)	142330(13308)	2163(62)	3.81(0.04)	51558(2269)	1505(25)	3.65(0.04)
	60	Guinier Porod	150750(15481)	2344(73)	3.87(0.04)	439930(98804)	3191(204)	3.93(0.04)	370910(75301)	3182(182)	3.96(0.05)
	90	Guinier Porod	259090(42527)	2805(130)	3.91(0.04)	741760(287830)	3916(410)	3.91(0.05)	449340(127160)	3456(264)	3.97(0.05)
	120	Power law	5.2E-8(1.4E-8)	–	3.80(0.04)	3.2E-8(8.9E-9)	–	3.88(0.04)	2.8E-8(8.0E-9)	–	3.87(0.04)
Milk at pH 6.0	120	Power law	1.9E-7(5.6E-8)	–	3.21(0.04)	3.9E-7(1.1E-7)	–	3.10(0.04)	5.6E-8(1.7E-8)	–	3.42(0.05)

Fitted  $q$  range:  $0.0006\text{--}0.01 \text{ \AA}^{-1}$ . Fixed parameters: background:  $0.1 \text{ cm}^{-1}$ ,  $s$ : 0 (for spherical particles). The results are expressed as the mean (standard deviation).

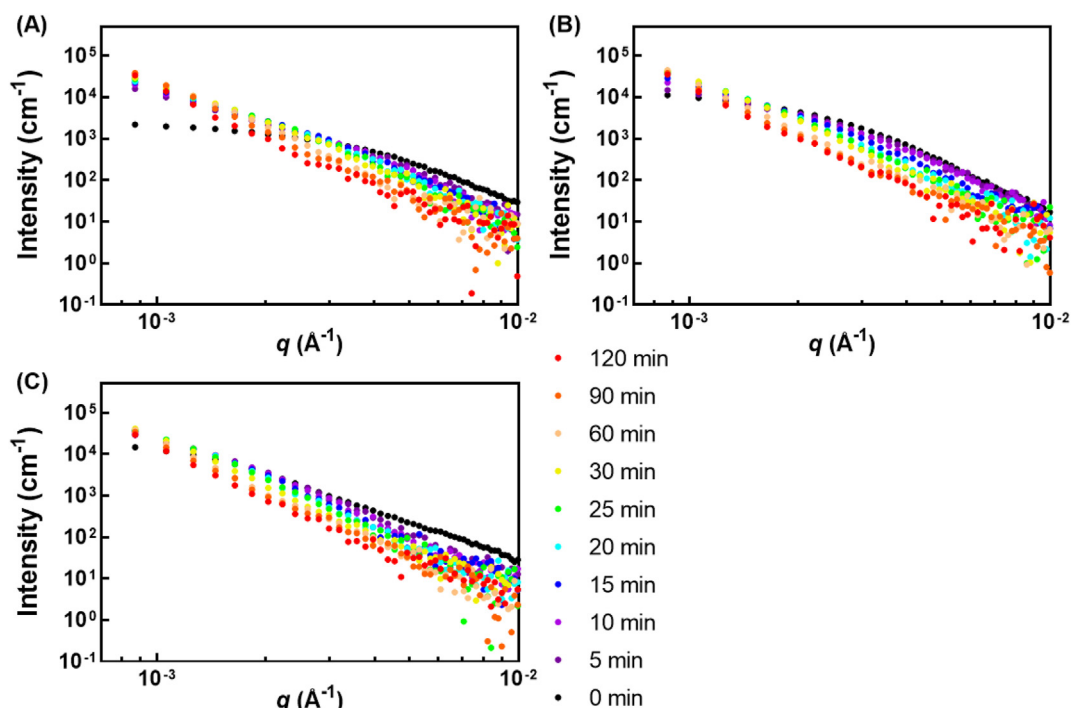


Fig. 5. Evolution of time-resolved SANS intensity data during coagulation of various milk samples (pH 6.3) up to 120 min (A: cow milk, B: sheep milk, C: goat milk).

rough surface fractal-like structures to form large aggregates in the milk curds (Chodankar, Aswal, Kohlbrecher, Vavrin, & Wagh, 2009; Vogtt, Javid, Alvarez, Sefcik, & Bellissent-Funel, 2011). The protein aggregate size was too large to be determined within the limited  $q$  range available, although ultra-SANS measurements would be valuable to extend the range to lower  $q$ .

A real-time SANS measurement was not conducted for samples at pH 6.0. However, the equilibrated milk curd, incubated for 120 min at 37 °C in an oven, was measured and is presented in Fig. 5. There are no Guinier shoulder features observed for all samples in the studied  $q$  range, due of the large size of aggregates in the samples. The power law exponents at  $0.0006 < q < 0.015 \text{ \AA}^{-1}$  of these samples were  $\sim 3.21$ ,  $\sim 3.10$  and  $\sim 3.42$  for the cow milk curd, sheep milk curd, and goat milk curd, respectively. Although Porod-type exponents can be interpreted in terms of the degree of surface roughness, in the current study, such an approach has not been

pursued as it is more likely that the scattering is a manifestation of a broad size distribution of aggregates (Schmidt, 1982). Based on the fitting parameters (Table 5), there was a significant decrease in the power law exponent when comparing the pepsin-induced curd at pH 6.3 and 6.0. The difference in power law exponent may be related to either surface roughness (Martin & Hurd, 1987) or associated with changes in size distribution of the aggregates (Schmidt, 1982) (although the aggregate size at 120 min could not be determined in the studied  $q$  range).

#### 4. Conclusions

At the same pepsin-to- $\kappa$ -casein ratio, the hydrolysis of  $\kappa$ -casein in sheep milk was most rapid, followed by cow milk  $\kappa$ -casein and goat milk  $\kappa$ -casein at both pH 6.3 and pH 6.0. The consequent coagulation processes of cow, goat and sheep milk were also different. Sheep milk coagulated when  $\sim 64\%$  of the  $\kappa$ -casein was hydrolyzed at pH 6.3 and when  $\sim 59\%$  of the  $\kappa$ -casein was hydrolyzed at pH 6.0; sheep milk coagula possessed the highest firmness and densest structure. The coagulation of goat milk was slow and required the highest extent of hydrolysis of  $\kappa$ -casein, namely  $\sim 90\%$  at pH 6.3 and  $\sim 86\%$  at pH 6.0. The curd formed from goat milk was softer (lower storage modulus), and the microstructure contained more large pores with fewer crosslinks between the protein aggregates compared to cow and sheep curds. The curd microstructure varied across different milk species at the same casein concentration. Such differences in the coagulation processes in curd formation are not only due to the casein content, but also related to other physicochemical properties of different types of milk. Such factors could include differences in  $\kappa$ -casein chemistry (e.g., degree of glycosylation, amino acid sequences), casein micelle size, casein micelle density (hydration) and mineralization. The fundamental insights generated from this study provide an improved understanding of pepsin-induced coagulation behavior of milk from different species.

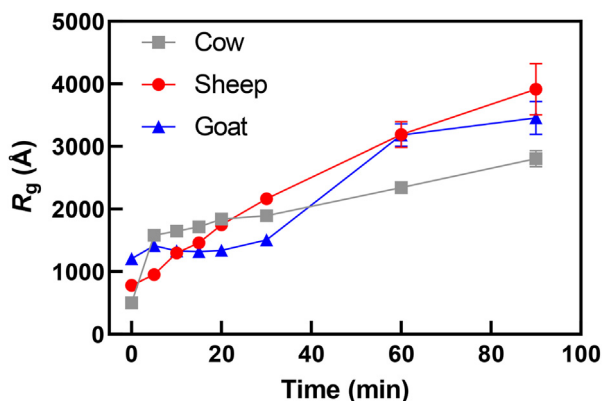


Fig. 6. Evolution of  $R_g$  (radius of gyration) of various milk samples (pH 6.3) after the addition of pepsin (associated with Fig. 5, fitted with Guinier–Porod model).

## CRediT authorship contribution statement

**Mengxiao Yang:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Aiqian Ye:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Elliot Paul Gilbert:** Writing – review & editing, Supervision, Methodology, Data curation. **Zhi Yang:** Writing – review & editing, Supervision, Methodology. **David W. Everett:** Writing – review & editing, Supervision. **Harjinder Singh:** Writing – review & editing, Supervision, Funding acquisition.

## Declaration of competing interest

None.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.idairyj.2024.105898>.

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